Novel Reduction-Responsive Cross-Linked Polyethylenimine Derivatives by Click Chemistry for Nonviral Gene Delivery

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Novel reducible disulfide-containing cross-linked polyethylenimines (PEI-SS-CLs) were synthesized via click chemistry and evaluated as nonviral gene delivery vectors. First, about four azide pendant groups were introduced into a low-molecular-weight (LMW) PEI (1.8 kDa) to get an azide-terminated PEI. Then, click reaction between a disulfide-containing dialkyne cross-linker and the azide functionalized LMW PEI resulted in a high-molecular-weight disulfide-containing cross-linked PEI composed of LMW constitute via a reducible cross-linker. The synthesized polymers were characterized by ^{1}H NMR, FTIR, and size-exclusion chromatography (SEC). It was shown that the obtained disulfide-containing cross-linked PEIs were able to condense plasmid DNA into positively charged nanoparticles. The degradation of the disulfide cross-linked polymers PEI-SS-CLs induced by DTT was confirmed by a gel retardation assay and SEC analysis. In vitro experiments revealed that the reducible PEI-SS-CLs were less cytotoxic and more effective in gene transfection (in both the presence and absence of serum) than the control nondegradable 25-kDa PEI. This study demonstrates that a reducibly degradable cationic polymer composed of LMW PEI cross-linked via a disulfide-containing linker possesses both higher gene transfection efficiency and lower cytotoxicity than PEI (25 kDa). These polymers are therefore attractive candidates for further in vivo evaluations.

INTRODUCTION

Gene therapy is a promising approach to treat human genetic and acquired diseases. The key to the success of gene therapy is the availability of safe and efficient vector systems (*1, 2*). Cationic polymers, such as poly(L-lysine) (*3*), chitosan and its derivatives (*4, 5*), poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) (*6*−*8*), and poly(ethylene imine) (PEI) (*9*), have been widely explored as nonviral gene vectors. Among them, PEI has been regarded as the "gold" standard showing relatively high transfection efficiency, particularly in vitro, due to the proton sponge effect (*10, 11*). Although transfection efficiency of PEI-based complexes increases with PEI molecular weight (*12*), cytotoxicity of PEI also simultaneously increases. High-molecular-weight (HMW) PEIs, such as 25 kDa PEI, not only are highly effective in gene transfection, but also exhibit significant cytotoxicity. Low molecular weight (LMW) PEIs, such as 1.8 kDa PEI, are less cytotoxic but less efficient (*12, 13*).

To deal with this dilemma between transfection efficiency and cytotoxicity, biodegradable cationic polymers have been designed with either hydrolytically degradable ester groups (*8, 14*) or reducibly degradable disulfide linkages (*15*−*19*). It is expected that these polymers complexed with plasmid DNA can be degraded into less toxic low-molecular-weight structures after cellular uptake, by which the polymers lose their DNA binding capacity resulting in DNA release inside target cells. Generally, ester linkages have a pH-dependent hydrolysis with half-life time of hours to days. In contrast, disulfide linkages have been shown to be stable in blood circulation (*20*), and their degradation is rapid (minutes to hours) in the presence of reductive 1,4-dithio-DL-threitol (DTT) or glutathione (GSH) mimicking the reductive intracellular environment (*20, 21*). Therefore, the introduction of disulfide bridges in the polymer main chain (*22*−*26*) or in the cross-links of polymers (*27, 28*) has been investigated for the design of polymeric gene delivery vectors (*8*). Gopferich et al. (*29*) recently published an excellent review on the redox-sensitive, disulfide-based carriers for delivery of nucleic acids. Lee et al. (*27*) first synthesized reducible PEI derivatives using 0.8 kDa PEI reacting with homobifunctional cross-linking reagents, dithiobis(succinimidylpropionate) (DSP), or dimethyl-3,3′-dithiobis-propionimidate-2HCl (DTBP). The resulting polymers showed higher transfection efficiency compared to the parent 0.8 kDa PEI and reached transfection levels close to that of 25 kDa PEI. In a similar way, 1.8 kDa PEIs cross-linked with DTBP were able to condense DNA, and the formed polyplexes were sensitive to the GSH with significantly low cytotoxicity (*30*). Also, 5-kDa PEIs cross-linked with DSP formed stable complexes with DNA which could be destabilized by a redox trigger (*31*). Kloeckner et al. (*32*) studied a library of 37 soluble polycations synthesized by oligomerization of LMW oligoamines with different cross-linkers. They concluded that the polyplexes based on 0.8 kDa PEIs cross-linked with DSP or DTBP showed negligible toxicity and efficient reporter gene transfection at high N/P ratios. Goepferich et al. (*33*) described that linear PEIs with molecular weight of 2.6, 3.1, or 4.6 kDa cross-linked with DSP or boc-cystine resulted in branched structures. Using seven different cell lines, they showed a high transfection efficiency (about 60%) and a high

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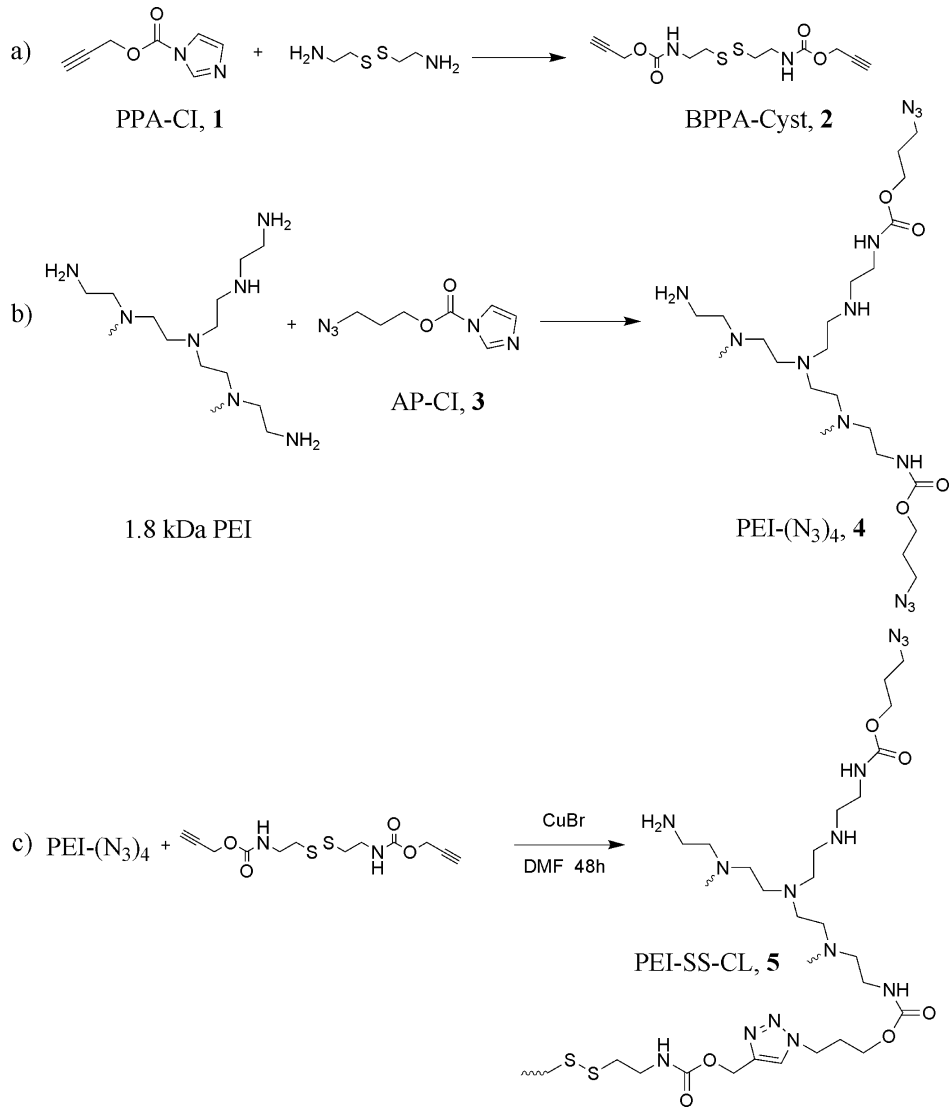
Scheme 1. Synthetic Route Towards Reducible Disulfide-Contained PEI Derivatives (PEI-SS-CLs)



cell viability (>90%) of polyplexes based on the reversibly cross-linked linear PEI polymers, which was better than seven commercial transfection reagents including SuperFect, Lipofectamine, and JetPEI. It was also reported (*34*−*36*) that the disulfide cross-linked PEI derivatives using LMW PEI and DSP were highly effective as gene delivery vectors and hardly showed cytotoxicity. However, the cross-linking reaction between PEI and DSP (or DTBP) can easily lead to the formation of microgels resulting in poorly controlled molecular weight and a low yield (*35*). Peng et al. (*15*) used methylthiirane reacting with low-molecular-weight PEI to get thiolated PEIs (PEI-SHx), which were cross-linked into higher-molecular-weight PEI by oxidation of the thiol groups avoiding significantly changing the acid−base property of the PEI. However, no reducible degradation was obviously observed. They claimed that the majority of disulfide cross-links are intramolecular when the thiolation degree is high.

Recently, "click" chemistry has rapidly become a very popular method for polymer synthesis and modification due to the high selectivity and high fidelity (*8, 37, 38*). However, no papers using click chemistry for PEI cross-linking have been published so far. In this study, a new strategy for synthesis of disulfide cross-linked polyethylenimine (PEI-SS-CL) via click reaction is investigated, as depicted in Scheme 1. First, a LMW PEI (1.8 kDa), which has low transfection efficiency and low cytotoxicity, was modified with azide pendant groups. Then, this azide-functional PEI was reacted with disulfide-containing dialkyne cross-linker through click chemistry to obtain a reducible PEI derivative with high molecular weight. Click chemistry allows easy coupling of poly(ethylene glycol) (PEG) shielding, targeting ligands and fluorescent groups into the polyplexes for further in vivo evaluation. This paper describes the synthesis and reduction-triggered degradation of the disulfide-containing cross-linked PEI polymers and the gene transfection activities of the corresponding polyplexes.

EXPERIMENTAL PROCEDURES

Materials. Branched polyethylenimine (PEI) with molecular weight of 1.8 kDa was purchased from Alfa Aesar, and branched PEI (25 kDa) was from Sigma-Aldrich. 1,1′-Carbonyldiimidazole (CDI) (Shanghai Medpep Co., Ltd., China), propargyl alcohol (Wuhan FengFan Chemical Co., Ltd., China), cystamine dihydrochloride (Jiangsu Jintan Medicine Chemical Factory, Jiangsu, China), 1,4-dithio-DL-threitol (DTT, Shanghai Regal Biotechnology Company, China), and 3-bromopropanol (Shanghai Zhuo Rui Chemical Co., China) were used as received. *N*,*N*′-Dimethylformamide (DMF) was obtained from Shanghai Chemical Reagent Co., China, and used after distillation under reduced pressure. Plasmid pcDNA3-Luc in TE buffer with concentration of 5.0 mg/mL was from Plasmid Factory, Germany. Plasmid pEGFP encoding a red-shifted variant of wild-type green

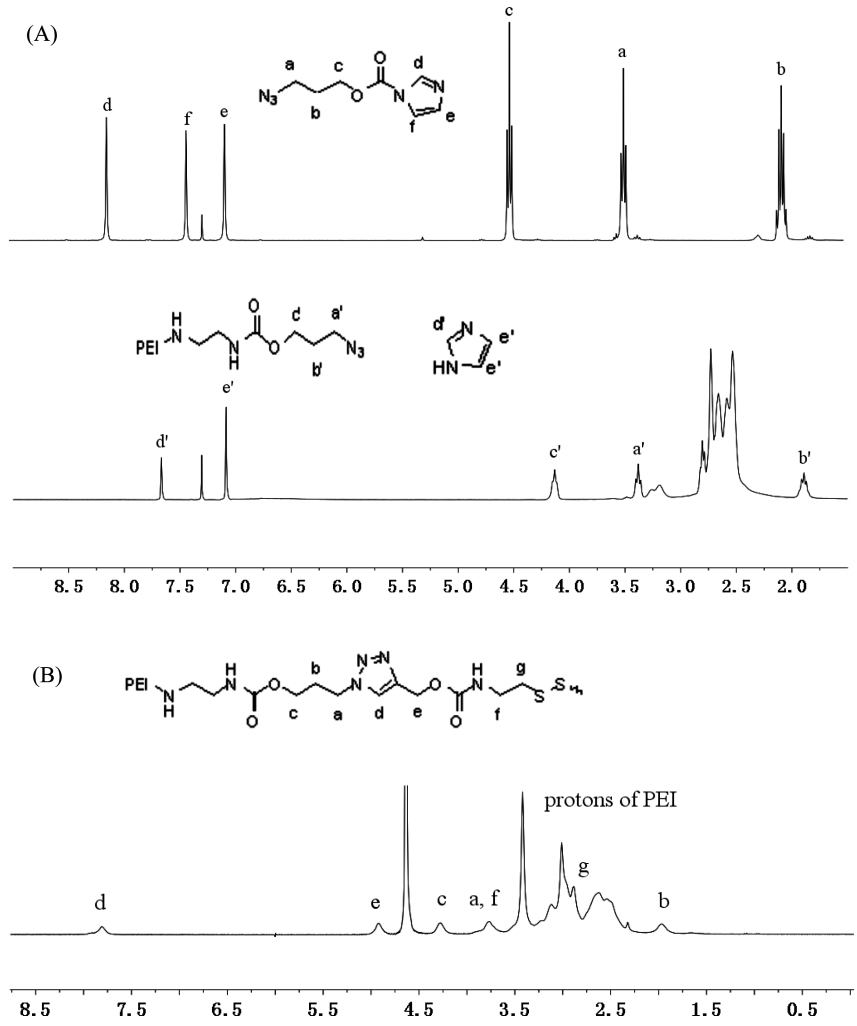


Figure 1. (A) ^{1}H NMR spectra of AP-CI and PEI-$(N_3)_4$ (with imidazole) in $CDCl_3$. (B) ^{1}H NMR spectra of PEI-SS-CL1 in D_2O.

fluorescent protein (GFP) was purchased from Clontech, Mountain View, CA, USA, and was transformed in *E. coli* DH5α and propagated in Luria−Bertani (LB) medium at 37 °C overnight. Then, the plasmids were purified by means of EndoFree plasmid purification as described previously (*39*). The purified plasmids were diluted with TE buffer solution and stored at −20 °C. The purity and concentration of plasmids were determined by ultraviolet (UV) absorbance at 260 and 280 nm. All other chemicals were analytical grade and used as received.

Synthesis of Disulfide-Containing Cross-Linked Polyethylenimine (PEI-SS-CL). *Synthesis of Bis[(Propargyl Carbamate)-Ethyl] Disulfide (BPPA-Cyst,* **2***).* Propargyl ester of carbonylimidazole (PPA-CI, **1**) was synthesized from 1,1′-carbonyldiimidazole (CDI) and propargyl alcohol (PPA) as described previously (**8**). Cystamine dihydrochloride was neutralized by 4 M NaOH and extracted with dichloromethane to yield cystamine. Then, BPPA-Cyst was synthesized from PPA-CI with the cystamine, as shown in Scheme 1. Briefly, cystamine (4.40 g, 28.52 mmol) and PPA-CI (6.93 g, 46.2 mmol) were dissolved in 50 mL dichloromethane and subsequently stirred at room temperature for 24 h. After evaporation of dichloromethane, 100 mL of 1.0 M NaH_2PO_4 (pH 4.0) was added. This aqueous solution was extracted with ethyl ether (40 mL, 3 times) to obtain, after evaporation of the solvent, BPPA-Cyst with a yield of 80.2%. ^{1}H NMR in $CDCl_3$ (Supporting Information Figure SI1): δ (ppm) 2.48 (s, 2H, ***CH***≡C), 2.82 (t, 4H, S-***CH$_2$***-C), 3.51 (app. quartet, 4H, NH-***CH$_2$***-C), 4.69(s, 4H, O-***CH$_2$***-C≡C), 5.31(s, 2H, ***NH***-C═O). IR analysis of the resulting product showed an absorption peak at 2126 cm^{-1} corresponding to the alkyne group (see Supporting Information Figure SI2, IR spectrum: 3293, 3061, 2943, 2126 ($\nu_{C\equiv C}$), and 1709 cm^{-1}).

Synthesis of 3-Azidopropyl Ester of Carbonylimidazole (AP-CI, **3***).* 3-Azidopropanol was synthesized according to the literature (*40*) with some modification. Briefly, 3-bromopropanol (3.01 g, 21.65 mmol) and sodium azide (5.62 g, 86.46 mmol) were dissolved in a mixture of 25 mL water and 10 mL acetone, stirred under reflux for 24 h in an oil bath of about 80 °C. After evaporation of acetone, the product was extracted with dichloromethane (25 mL, 3 times). The organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated to obtain colorless oil with a yield of 87.6%. ^{1}H NMR ($DCCl_3$): δ (ppm) 1.80 (app. quint, 2H, C-***CH$_2$***-C), 3.41 (t, 2H, ***CH$_2$***-N_3), 3.70 (t, 2H, ***CH$_2$***-O).

AP-CI (**3**) was synthesized from the obtained 3-azidopropanol with carbonyldiimidazole (CDI). Briefly, a dry, round-bottomed flask was charged with CDI (4.80 g, 29.60 mmol) and 40 mL dichloromethane yielding a suspension. Next, 3-azidopropanol (1.50 g, 12.40 mmol) was added dropwise under vigorous stirring while the reaction mixture turned into a clear solution. After 4 h reaction at room temperature, the solution was washed three times with water. The organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated to obtain colorless oil with a yield of 74.3%. ^{1}H NMR in $CDCl_3$ as shown in Figure 1A: δ (ppm) 2.05 (app. quint, 2H, C-***CH$_2$***-C), 3.47 (t, 2H, N_3-***CH$_2$***), 4.50 (t, 2H, ***CH$_2$***-O), 7.06 (s, 1H, C═***CH***—N═), 7.40 (s, 1H, N—***CH***═C), 8.12 (s, 1H, N—***CH***═N).

Table 1. Characteristics of the Synthesized Disulfide Cross-Linked PEI-SS-CLs

polymers	molar feed ratios of alkyne/azide	yield(%)	M_w (kDa)	PDI
PEI-SS-CL1	1:1	59	9.4	3.12
PEI-SS-CL2	1:2	49	14.2	1.85

*Synthesis of Azide-Terminated PEI [**4**, PEI-$(N_3)_4$].* AP-CI (2.17 g, 11.13 mmol) dissolved in chloroform (20 mL) was slowly dropped (around 2 h) into a solution of 1.8 kDa PEI (5.00 g, 2.78 mmol, 29 mmol of primary amine groups, assuming that the primary amine content of the 1.8 kD PEI is 25% of the total amines (*13*) in chloroform (100 mL). Next, the reaction mixture was refluxed using an oil bath (75 °C) for 10 h. Chloroform was removed by evaporation under reduced pressure yielding a yellow liquid which was subsequently dissolved in $CDCl_3$ and characterized by ^1H NMR. No unreacted AP-CI was detected as shown in Figure 1A. The average number of azide groups per PEI molecule determined by ^1H NMR was close to 4. IR analysis of the resulting product showed an absorption peak at 2099 cm^{-1} corresponding to the asymmetric stretching vibration of the azide group (see Supporting Information Figure SI2, IR spectrum (neat): 2944, 2837, 2933, 2099 (ν_{N3}), and 1700 cm^{-1}).

Synthesis of Disulfide Cross-Linked PEI-SS-CL (5) via Click Reaction. Azide-functional PEI (PEI-$(N_3)_4$, 0.75 g, 1 equiv azide groups) and BPPA-cyst (0.16 or 0.08 g, 1 or 0.5 equiv alkyne groups) were dissolved in DMF, and CuBr (1 equiv) as catalyst was added under a nitrogen atmosphere. The reaction mixture was stirred at 50 °C for 2 days. The resulting solution was purified by dialysis against water (dialysis tube, MWCO 3.5 kDa), and the formed disulfide cross-linked PEI derivatives PEI-SS-CL1 and PEI-SS-CL2 were collected after freeze−drying. The structure of the PEI-SS-CLs was analyzed by ^1H NMR and FTIR. The molecular weights were obtained by size-exclusion chromatography−multiangle light scattering (SEC-MALLS) as described below.

Characterizations. ^1H nuclear magnetic resonance (NMR) spectra were measured with a Varian Unity 300 MHz spectrometer using $CDCl_3$ or D_2O as solvent. Fourier transformed infrared (FTIR) spectra were recorded on a Lambda Bio40 UV−vis spectrometer (Perkin-Elmer). The molecular weights and the molecular weight distributions of the polymers were evaluated by a SEC-MALLS system consisting of a Waters 2690D separations module, a Waters 2414 refractive index detector (RI), and a Wyatt DAWN EOS MALLS detector. Two chromatographic columns (Shodex OHpak SB-803 and SB-802.5, Showa Denko, Japan) with a precolumn (Shodex SB-G) were used in series. A sodium acetate (NaAc) solution was prepared by dissolving a calculated amount of sodium acetate in reverse osmosis water (0.3 M), and the pH was adjusted to 4.4 with acetic acid (*41*). A mixture of 70% 0.3 M NaAc solution, pH 4.4, and 30% acetonitrile was used as the eluent at a flow rate of 0.6 mL/min. The eluent was filtered through a 0.22 μm HPLC filter and degassed prior to use by ultrasound. The data were processed with *Astra* software (Wyatt Technology).

The reductive degradation of the disulfide cross-linked polymer PEI-SS-CL triggered by DTT was evaluated by SEC-MALLS. The polymer PEI-SS-CL (5.0 mg) was dissolved in the mobile phase (0.9 mL) followed by the addition of 0.1 mL DTT in water (1.0 mol/L). After incubation at 37 °C for 2 h, the molecular weight of the degraded polymer was determined by SEC-MALLS.

Cell Culture. Human embryonic kidney transformed (293T) cells and human cervix carcinoma (HeLa) cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 ug/mL streptomycin at 37 °C under a humidified atmosphere of 95% air and 5% CO_2.

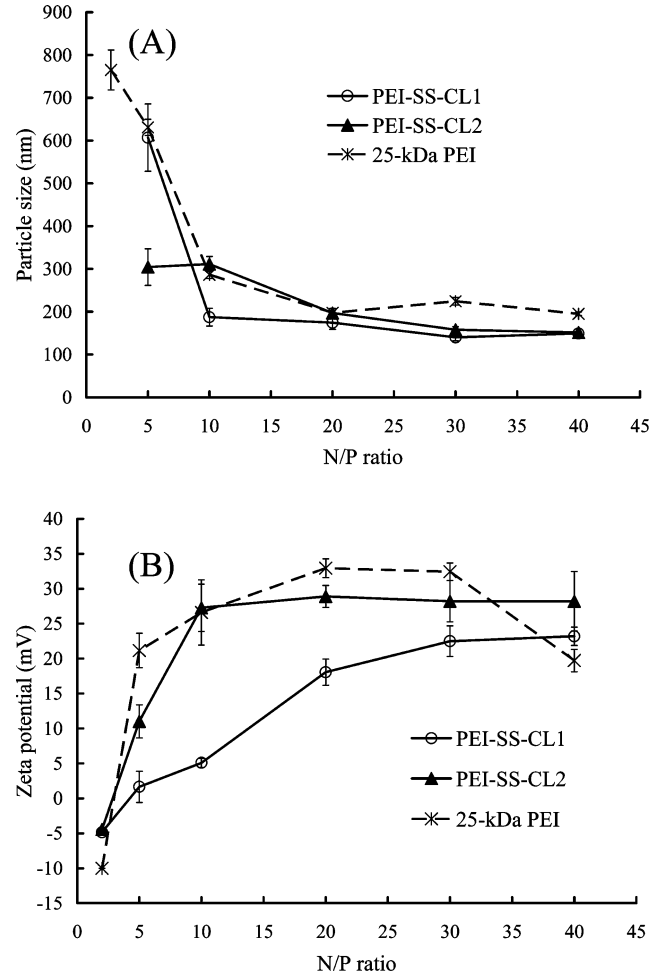


Figure 2. Size by DLS (A) and zeta potential (B) of the PEI/DNA polyplexes at various N/P ratios. Error bars are standard deviations (SD) of 3 measurements.

Cytotoxicity Assay. The cytotoxicity of the different polymers was studied using the MTT assay (*39*) on 293T cells. The cells were seeded into 96-well plates at a density of 3000 cells/well, and 100 μL DMEM containing 10% FBS was added. The cells were allowed to grow at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 for 48 h. Thereafter, the medium was replaced with 100 μL of fresh medium and 100 μL solutions of PEI-SS-CLs, 25 kDa PEI and 1.8 kDa PEI, were added (final polymer concentrations ranging from 0.01 to 0.20 mg/mL). The cells were incubated for 48 h, and the medium containing polymer solution was replaced with 200 μL fresh medium. Next, the MTT reagent (20 μL in PBS, 5 mg/mL) was added to each well for further 4 h incubation at 37 °C. Then, the medium was removed and 100 μL of DMSO was added to dissolve the formed formazan crystals. The absorbance at 570 nm was recorded using a Microplate Reader (BIO-RAD 550). The relative cell viability (mean ± SD, $n = 4$) was calculated as cell viability = $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$, where OD_{sample} is the absorbance of solution with cells treated by polymers, $OD_{control}$ is the absorbance for untreated cells (without polymer), and OD_{blank} is the absorbance without cells.

Formation of PEI-SS-CL/DNA Polyplexes and Agarose Gel Retardation Assay. PEI-SS-CL/DNA complexes (N/P ratios ranging from 3 to 9) were prepared by addition of 5 μL polymer solution with designed concentrations in 150 mM NaCl

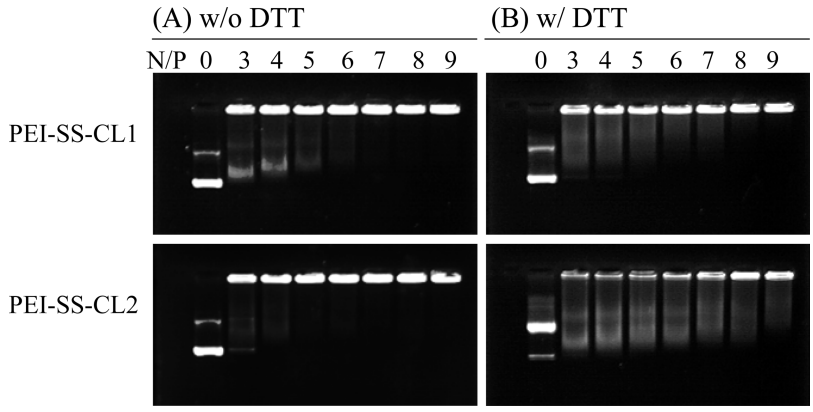


Figure 3. Agarose gel electrophoresis retardation assay of PEI-SS-CLs/DNA complexes at different N/P ratios (A) without DTT and (B) with 10 mM DTT.

(pH 7.4) to 1 μL of pcDNA3-Luc plasmid DNA (100 ng/μL in TE buffer). The resulting polyplex dispersions were incubated at 37 °C for 30 min and were then analyzed by electrophoresis in a 0.7% (w/v) agarose gel containing GelRed and in Tris-acetate (TAE) running buffer at 80 V for 60 min. DNA bands were visualized with a UV (254 nm) illuminator and photographed with a Vilber Lourmat imaging system.

The DTT triggered reductive degradation of PEI-SS-CL based polyplexes was also evaluated by agarose gel electrophoresis. To 1 mL of dispersions of PEI-SS-CL/DNA polyplexes prepared at N/P ratios ranging from 3 to 9, 100 μL of 100 mM DTT in water was added. The polyplexes were incubated at 37 °C for 30 min and were then analyzed by electrophoresis in a 0.7% (w/v) agarose gel containing GelRed and in TAE running buffer at 80 V for 60 min.

Particle Size and ζ-Potential Measurements. Polyplexes were prepared by the addition 90 μL of a PEI solution with designed concentrations (in 150 mM NaCl, pH 7.4) to 10 μL of pcDNA3-Luc plasmid DNA solution (0.1 mg/mL in 40 mM Tris-HCl buffer). The amount of polymer used was calculated on the basis of chosen N/P ratios, nitrogen atoms of the polymer over phosphates of DNA. After the complexes were incubated at 37 °C for 30 min, the polyplex dispersion was diluted with water to 1 mL prior to measurement. Particle size and ζ-potential were measured by Nano-ZS ZEN3600 (Malvern Instruments) at 25 °C.

In Vitro Transfection Activity of Polyplexes. *Luciferase Assay.* Transfection activity of polyplexes based on pcDNA3-Luc plasmid, complexed with PEI-SS-CLs, 25-kDa PEI or 1.8-kDa PEI, was evaluated in 293T and HeLa cells. The cells were seeded at a density of 70 000 cells/well in a 24-well plate. Subsequently, 1 mL of DMEM containing of 10% FBS was added, and the cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. Next, the medium in each well was replaced by 0.9 mL of serum-free DMEM. The polymer/DNA polyplexes (100 μL, N/P ratios ranging from 10 to 50) prepared as described above were then added and incubated with cells for 4 h at 37 °C. The medium was replaced by fresh DMEM containing 10% FBS, and the cells were incubated for 48 h. Thereafter, the medium was removed, and the luciferase assay was performed according to manufacturer's protocols. Relative light units (RLUs) were measured with chemiluminometer (Lumat LB9507, EG&G Berthold, Germany). The total protein was measured according to a BCA protein assay kit (Pierce). Luciferase activity was expressed as RLU/mg protein.

Transfection experiments in 293T cells in the presence of serum were carried out. The protocol was the same as described above, except that the cell medium of DMEM with 10% FBS was used instead of the serum-free DMEM medium. All the transfection assays were carried out in triplicate.

Green Fluorescent Protein Assay. Transfection activity of complexes of PEI-SS-CLs with pEGFP plasmid was evaluated in 293T cells. The 293T cells were seeded at a density of 70 000 cells/well in a 24-well plate. Subsequently, 1 mL of DMEM with 10% FBS was added, and the cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. Next, the cells were incubated for 4 h at 37 °C, followed by the addition of polyplexes of various N/P ratios prepared as described above. After transfection for 48 h, the cells were directly observed by an inverted microscope (Olympus IX 70). The microscopy images were obtained at the magnification of 100× and recorded using *CoolSNAP-Pro* (v 4.5.1.1) software.

RESULTS AND DISCUSSION

Synthesis and Characterization of Disulfide-Containing PEI-SS-CL. The two-step synthetic route of disulfide cross-linked polyethylenimine (PEI-SS-CL) via click reaction is illustrated in Scheme 1. First, azide pendant groups were introduced into a low-molecular-weight (LMW) PEI (1.8 kDa) to obtain PEI-$(N_3)x$, in which x stands for the average number of azide groups per PEI molecule. Second, the azide-functional PEI was reacted in DMF with the disulfide-containing dialkyne cross-linker BPPA-Cyst, through click chemistry with CuBr as catalyst, to obtain a reducible PEI derivative with high molecular weight.

Azide-functionalized LMW PEI was synthesized by coupling of activated azidopropanol, 3-azidopropyl ester of carbonylimidazole (AP-CI, **3**, Scheme 1), to PEI. ^{1}H NMR analysis of the obtained product (Figure 1A) shows the absence of the starting compound AP-CI (comparing the proton NMR spectra of AP-CI and PEI-$(N_3)_4$ in Figure 1A, no peaks at 8.12 (d) and 7.40 ppm (f) for PEI-$(N_3)_4$, the peaks at 4.50 (c), 3.47 (a), and 2.05 ppm (b) in AP-CI shifting to 4.09 (c′), 3.34 (a′), and 1.85 ppm (b′) for PEI-$(N_3)_4$, respectively). Also, imidazole (with one proton peak at 7.66 and two protons at 7.04 ppm) was detected in the resulting product. The IR spectrum of the resulting product shows an absorption peak at 2099 cm^{-1} corresponding to the asymmetric stretching vibration of the azide group (see Supporting Information Figure SI2). From NMR and IR analysis, it is therefore concluded that azide-functionalized PEI has been obtained. The average number of azide groups per PEI molecule (x in PEI-$(N_3)x$) was calculated on the basis of the ratio of the integral from 2.30 to 2.90 ppm of the PEI protons to the protons of azidopropanol at 3.34 ppm (2*H*, next to azide group), assuming that the primary amine content of the PEI (1.8 kD) is 25% of the total amines (*13, 27*). ^{1}H NMR analysis shows that x in PEI-$(N_3)x$ is 4.1 when the AP-CI/PEI molar feed ratio was

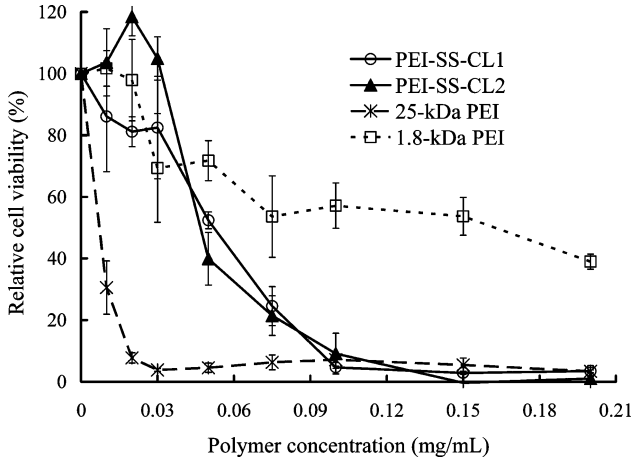


Figure 4. Cytotoxicity of cationic polymers PEI-SS-CLs and controls 1.8 kDa PEI and 25 kDa PEI to 293T cells after 48 h incubation (mean ± SD, $n = 4$).

Table 2. IC_{50} Values of PEI-SS-CLs, 1.8 kDa PEI and 25 kDa PEI

polymers	PEI-SS-CL1	PEI-SS-CL2	1.8-kDa PEI	25-kDa PEI
IC_{50} (μg/mL)	52	47	162	6

4, which indicates that AP-CI had reacted quantitatively with PEI. Because of the quantitative conversion, the reaction mixture only contains PEI-$(N_3)x$, imidazole, and solvent; no further purification was required for the next click reaction after evaporation removal of the solvent, chloroform.

The azide-functionalized LMW PEI-$(N_3)_4$ was cross-linked with disulfide-containing dialkyne BPPA-Cyst by click reaction in DMF at 50 °C for 48 h, using CuBr as catalyst, to obtain reducible HMW PEI derivative, PEI-SS-CL. Two disulfide cross-linked PEIs, named PEI-SS-CL1 and PEI-SS-CL2, were obtained with molar feed ratios of alkyne/azide of 1:1 and 1:2, respectively. Both polymers are water-soluble, which indicates that the cross-linking reactions between dialkynes and azide groups only take place within a definite number of LMW PEI molecules. However, with increasing cross-linking ratio (molar feed ratio alkyne/azide 1:0.75), microgels were formed. The soluble polymers, PEI-SS-CL1 and PEI-SS-CL2, were further investigated as gene vectors.

The structures of these soluble polymers were investigated by ^{1}H NMR and FTIR analysis. The ^{1}H NMR spectrum of PEI-SS-CL1 is shown in Figure 1B, from which it can be seen that a new peak appeared at 7.8 ppm (*H* of the triazole ring) indicating the formation of triazole groups by click reaction. The absorption peaks at 2099 cm^{-1} and 2126 cm^{-1} in the IR spectrum for polymer PEI-SS-CL1 are very weak (Supporting Information Figure SI2), which indicates that the azide groups reacted almost quantitatively with the alkyne groups.

The molecular weights and molecular weight distributions of the PEI-SS-CLs were determined by SEC-MALLS (Table 1). The weight average molecular weights of PEI-SS-CL1 and PEI-SS-CL2 were about 5 and 7 times that of the starting polymer PEI 1.8 kDa. With all ^{1}H NMR, FTIR, and SEC analysis results taken together, it can be concluded that the click reactions between BPPA-Cyst and PEI-$(N_3)_4$ were successful.

Characterization of PEI-SS-CL-Based Polyplexes. To be effective as gene delivery vectors, cationic polymers must possess the capacity to bind and condense plasmid DNA into nanoparticles which can be taken up by cells. The DNA binding and condensation properties of the starting LMW PEI (1.8 kD), the synthesized disulfide-containing PEI-SS-CLs and the branched PEI (25 kD), were investigated. It was shown by DLS measurements that 1.8 kDa PEI, even at an N/P ratio up to 50, was

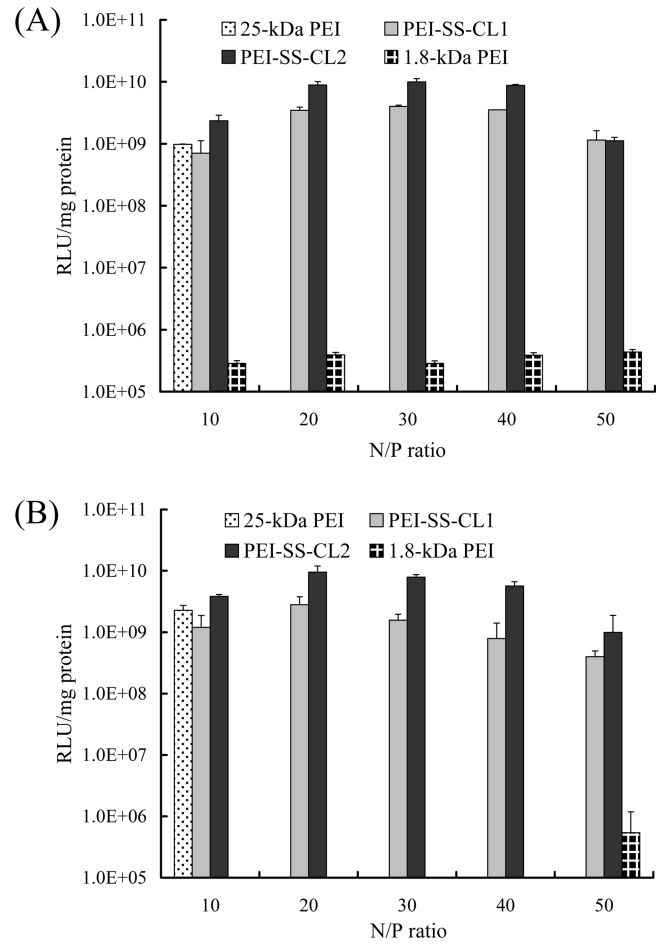


Figure 5. Luciferase expression of the PEI-SS-CL/DNA polyplexes at different N/P ratios with control 1.8 kDa PEI and 25 kDa PEI (at N/P ratio of 10) in serum-free DMEM in 293T (A) and HeLa cells (B).

unable to condense DNA. Importantly, at N/P ratios above 10, the two disulfide-containing PEI-SS-CLs prepared from the 1.8 kDa PEI were able to condense DNA into small particles with size of around 200 nm and a positive zeta potential of approximately +20 mV, similar to the 25 kDa PEI (Figure 2).

The gel retardation assay further confirmed that the two disulfide-containing PEI-SS-CLs can bind plasmid DNA efficiently and retard DNA migration at N/P ratios above 7 (Figure 3). In agreement with DLS analysis, 1.8 kDa PEI was unable to bind DNA even at an N/P ratio up to 50 (data not shown).

Reductive Degradation of the PEI-SS-CL. The DTT-induced degradation of the disulfide cross-linked PEI-SS-CLs was investigated using the agarose gel retardation assay and SEC analysis. The SEC traces of PEI-SS-CL1 and PEI-SS-CL2 in the absence and presence of DTT (Supporting Information Figure SI3) clearly show that the disulfide cross-linked polymers PEI-SS-CLs were responsive to the reductive agent DTT. After incubation with DTT for 2 h at room temperature, the weight average molecular weight of PEI-SS-CL1 decreased from 9.4 kDa (molecular weight distribution was 3.1) to 2.1 kDa (molecular weight distribution was 3.3), which is very close to M_w of PEI (1.8 kDa). Agarose gel electrophoresis (Figure 3B) shows the presence of free DNA in PEI-SS-CL/DNA polyplexes incubated with 10 mM DTT (30 min), which indicates that in a reductive environment PEI-SS-CL1 polyplexes released DNA.

In Vitro Cytotoxicity. The MTT assay for 293T cells was carried out to determine the cytotoxicity of disulfide-containing PEI-SS-CLs; 1.8 kDa PEI and 25 kDa PEI were used as controls. Figure 4 shows that the PEI-SS-CLs had a lower cytotoxicity than 25k Da PEI. The half inhibitory concentrations (IC_{50}) of

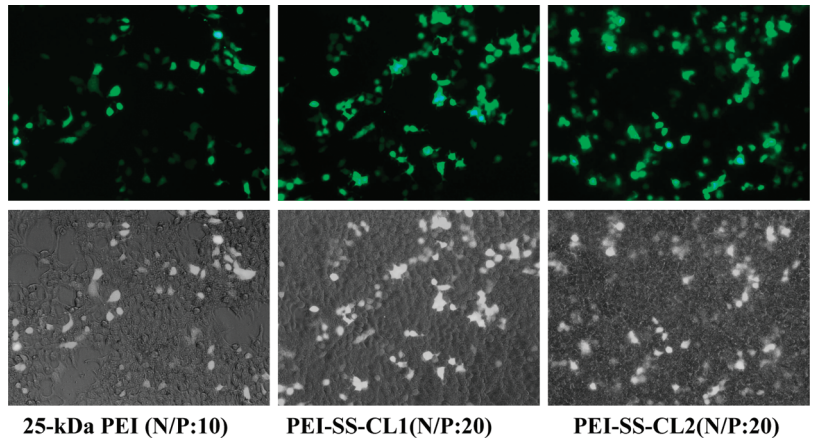


Figure 6. Fluorescent and bright-field images of enhanced green fluorescent protein expression in 293T cells for PEI-SS-CL based polyplexes (N/P ratio 20) with control 25 kDa PEI (N/P ratio 10). The images were obtained at magnification of 100×.

the polymers are summarized in Table 2 and are 52 (PEI-SS-CL1) and 47 μg/mL (PEI-SS-CL2), about 8−10× higher than that of 25 kDa PEI (6 μg/mL). The low cytocompatibility of HMW PEI is probably due to its high binding and aggregation on the cell surface and internalization, which results in significant necrosis (*13*). The low cytotoxicity of disulfide cross-linked PEI-SS-CLs indicates that, after cellular binding and internalization, they are intercellularly degraded, triggered by gluthatione, into relatively low-toxicity products.

In Vitro Transfection. The transfection activity of PEI-SS-CLs based polyplexes was evaluated in 293T and HeLa cells using the plasmid pcDNA3-Luc (expressing luciferase) and pEGFP (expressing green fluorescence) as reporter genes. Branched 25 kDa PEI and 1.8 kDa PEI, which were used for preparation of PEI-SS-CLs, were used as controls.

Figure 5 shows that PEI-SS-CL polyplexes had significantly higher transfection activity in both 293T and HeLa cells than those based on 1.8 kDa PEI. Also, incubation of cells with the PEI-SS-CL2 polyplexes resulted in about 5−10× higher luciferase expression in HeLa and in 293T cells, respectively, than polyplexes based on 25 kDa PEI, although the molecular weight of PEI-SS-CL2 is lower than that of branched PEI (25 kDa). The reason may be that PEI-SS-CLs polyplexes are degraded through cleavage of disulfide bonds inside the cells into nontoxic small PEI; DNA would be released selectively inside the cells to mediate efficient gene transfection (*15, 25, 27*). The transfection activity of the PEI-SS-CL2 polyplexes was higher than those of PEI-SS-CL1 at all N/P ratios studied, which can probably be ascribed to higher molecular weight of PEI-SS-CL2 .

The outstanding transfection activity of PEI-SS-CL polyplexes was visualized with a fluorescent microscope (Figure 6). The cells incubated with PEI-SS-CLs and 25-kDa PEI polyplexes showed many intracellular bright green fluorescent spots. As shown in Figure 6, substantial more fluorescent spots were observed using PEI-SS-CL1 and PEI-SS-CL2 polyplexes prepared at an N/P ratio of 20 than those based on 25-kDa PEI, which is in line with transfection data using the luciferase assay.

The transfection ability of the polyplexes of the disulfide cross-linked PEI-SS-CLs with plasmid pcDNA3-Luc in the presence of serum (10% FBS) was also investigated using 293T cells. As shown in Figure 7, the transfection efficiencies in 293T cells of the two PEI-SS-CL/DNA polyplex formulations were higher than that of the 25 kDa PEI based polyplexes in DMEM medium with 10% FBS. Especially, the PEI-SS-CL2 based polyplexes at an N/P ratio of 40 showed about an 8-fold increase in luciferase reporter gene expression compared to the control 25 kDa PEI polyplexes. This is in agreement with the reports

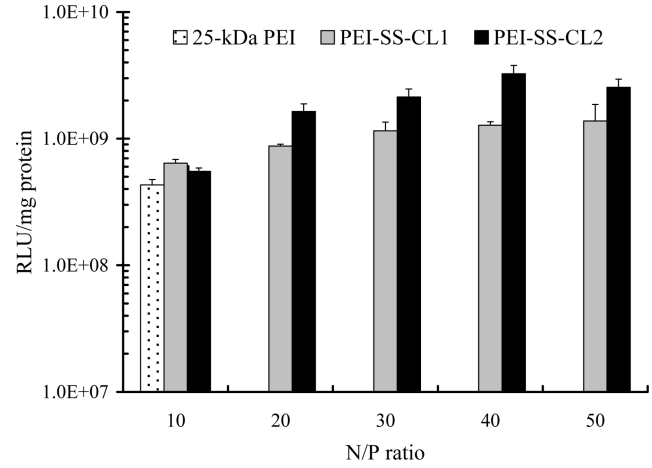


Figure 7. Luciferase expression of PEI-SS-CL/DNA polyplexes at different N/P ratios with control 25 kDa PEI (at N/P ratio of 10) in the presence of 10% FBS in 293T cells.

that the network poly(amino ester) (*42, 43*) and disulfide cross-linked PEI (*15*) based polyplexes showed very high transfection efficiency even in the presence of serum. One possible explanation is that the cross-linked polymers have outer shells with a large proportion of the groups exposed at the surface, which may facilitate multiple simultaneous interactions with the nucleic aid phosphates and fairly maintain the stability of polymer/DNA polyplexes in the presence of serum. The other possible reason might be the introduction of reducibly cleavable disulfide bonds. Thus, importantly, the polyplexes remain active in the presence of serum proteins, which encourages further in vivo evaluation.

CONCLUSIONS

The disulfide-containing cross-linked polyethylenimine derivatives (PEI-SS-CL1 and PEI-SS-CL2) were synthesized via click reaction between disulfide-containing dialkyne cross-linker BPPA-Cyst and azide-functionalized LMW PEI. The synthesized polymers were characterized by ^{1}H NMR, FTIR, and SEC. The DNA binding and condensation properties of PEI-SS-CL were investigated for the complexes at various N/P ratios by DLS, zeta potential, and agarose gel retardation assay. We showed that the disulfide-containing PEI-SS-CLs were able to bind plasmid DNA efficiently to yield small polyplexes. The degradation of the PEI-SS-CLs in the presence of DTT was confirmed by gel retardation assay and SEC analysis. In vitro experiments demonstrated that the reducible PEI-SS-CLs not

only had much lower cytotoxicity, but also posed superior transfection activity (in both the presence and absence of serum) as compared to the control nondegradable 25 kDa PEI. Our results imply that the obtained disulfide cross-linked PEI-SS-CLs might be suitable for further in vivo gene transfection. Future work will focus on coupling of poly(ethylene glycol) (PEG) shielding, targeting ligands and fluorescent groups in the polyplexes for further in vivo evaluation.

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Supporting Information Available: Supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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